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Insect Biochemistry and Molecular Biology 34 (2004) 1315–1328
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Expression and evolution of Δ^9 and Δ^{11} desaturase genes in the moth *Spodoptera littoralis*

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Received 5 August 2004; received in revised form 9 September 2004; accepted 24 September 2004

Abstract

Desaturation of fatty acids is a key reaction in the biosynthesis of moth sex pheromones. The main component of *Spodoptera littoralis* sex pheromone blend is produced by the action of Δ^{11} and Δ^9 desaturases. In this article, we report on the cloning of four desaturase-like genes in this species: one from the fat body (Sls-FL1) and three (Sls-FL2, Sls-FL3 and Sls-FL4) from the pheromone gland. By means of a computational/phylogenetic method, as well as functional assays, the desaturase gene products have been characterized. The fat body gene expressed a Δ^9 desaturase that produced (*Z*)-9-hexadecenoic and (*Z*)-9-octadecenoic acids in a (1:4.5) ratio, whereas the pheromone gland Sls-FL2 expressed a Δ^9 desaturase that produced (*Z*)-9-hexadecenoic and (*Z*)-9-octadecenoic acids in a (1.5:1) ratio. Although both Δ^9 desaturases produced (*Z*)-9-tetradecenoic acid from myristic acid, transformed yeast grown in the presence of a mixture of myristic and (*E*)-11-tetradecenoic acids produced (*Z*,*E*)-9,11-tetradecadienoic acid, but not (*Z*)-9-tetradecenoic acid. The Sls-FL3 gene expressed a protein that produced a mixture of (*E*)-11-tetradecenoic, (*Z*)-11-tetradecenoic, (*Z*)-11-hexadecenoic and (*Z*)-11-octadecenoic acids in a 5:4:60:31 ratio. Despite having all the characteristics of a desaturase gene, no function could be found for Sls-FL4.

Keywords: Pheromone; Desaturase; Fatty acid; Unsaturation; cDNA; Cloning

1. Introduction

The ability of cells to modulate the degree of unsaturation in their membranes is mainly determined by the action of fatty acid desaturases, which introduce unsaturated bonds at specific positions in their fatty acyl chain substrates (Tocher et al., 1998). Desaturases can be differentiated by the electron carrier that they require to catalyze the formation of a double bond and the activated form of the acyl substrate (McKeon and Stumpf, 1982; Wada et al., 1993; Jaworski, 1987; Macartney et al., 1994; Murata and Wada, 1995). Desaturase enzymes also differ in their selectivity both for substrate chain length and for the location of

Abbreviations: DMDS, dimethyldisulfide; GC/MS, gas chromatography coupled to mass spectrometry; MS, mass spectrometry; MTAD, 4-methyl-1, 2, 4-triazoline-3, 5-dione; MUFA, monounsaturated fatty acids; ORF, open-reading frame; PCR, polymerase chain reaction; RACE, rapid amplification cDNA ends; SD, selective medium; YPD, yeast extract/peptone/dextrose medium. Products are abbreviated as: 14:Acid, myristic acid; E11–14:Acid, (*E*)-11-tetradecenoic acid; E13–16:Acid, (*E*)-13-hexadecenoic acid; Z9–14:Acid, (*Z*)-9-tetradecenoic acid; Z9–16:Acid, palmitoleic acid; Z9–18:Acid, oleic acid; Z9, E11–14:Acid, (Z, E)-9, 11-tetradecadienoic acid; Z11–14:Acid, (*Z*)-11-tetradecenoic acid; Z11–16:Acid, (*Z*)-11-hexadecenoic acid; GenBank Data Bank Accession numbers: SlsZ9(18), AY362877; SlsZ9(16), AY362878; SlsZ/E11, AY362879; Sls-NF, AY362880

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doi:10.1016/j.ibmb.2004.09.003

existing double bonds in the fatty acyl chain (Shanklin and Cahoon, 1998).

Desaturation of acyl coenzyme-A (CoA) esters is a common reaction in sex pheromone biosynthetic pathways in Lepidoptera for production of various unsaturated pheromone-precursor acids (Tillman et al., 1999). This biotransformation is catalyzed by integral acyl-CoA desaturases that are biochemically and structurally similar to the ubiquitous Δ^9acyl-CoA desaturases of animals and fungi (Shanklin and Cahoon, 1998). The homology of lepidopteran pheromone desaturases with the integral membrane acyl-CoA desaturases was confirmed by the cloning and functional expression of the first Δ^{11} desaturase-encoding cDNA isolated from the pheromone glands of *Trichoplusia ni* (Knipple et al., 1998). Orthologs of this desaturase were later found in other moth species, including *Helicoverpa zea* (Rosenfield et al., 2001), *Ostrinia nubilalis* and *O. furnacalis* (Roelofs et al., 2002), *Epiphyas postvittana* (Liu et al., 2002a), *Choristoneura rosaceana* (Hao et al., 2002a, b), *Argyrotaenia velutinana* (Liu et al., 2002b) and *H. assulta* (Jeong et al., 2003). Cloned desaturases specifically involved in sex pheromone biosynthesis with novel regiospecificities include the Δ^{14}desaturase of *Ostrinia* species (Roelofs et al., 2002) and the Δ^{10}desaturase of *Planotortrix octo* (Hao et al., 2002a). Additionally, Δ^9desaturase-encoding transcripts have been characterized from the fat bodies and pheromone glands in these moth species (Liu et al., 1999; Rosenfield et al., 2001; Hao et al., 2002a, b; Liu et al., 2002a, b; Roelofs et al., 2002; Jeong et al., 2003).

These enzymes are found in two groups differing in their substrate preference (Roelofs et al., 2002). One group contains Δ^9 desaturases identified in fat body tissue and, except for noctuids, and produces more palmitoleic than oleic acid (16>18) as is common in other animal species. However, a more recently evolved Δ^9 desaturase producing more oleic than palmitoleic acid (18>16) is found in the fat body of the noctuid species studied and the pheromone gland of some other moth species.

Thus, unlike the metabolic Δ^9acyl-CoA desaturases, pheromone gland desaturases have been found to catalyze the formation of unique unsaturated fatty acyl-CoA esters with variable chain lengths, different locations of unsaturations and either the ordinary *Z* or the unusual *E* double bond geometry (Bjostad and Roelofs, 1981, 1983; Roelofs and Bjostad, 1984; Wolf and Roelofs, 1986; Lofstedt and Bengtsson, 1988; Foster and Roelofs, 1990; Martinez et al., 1990; Zhao et al., 1990; Jurenka et al., 1991; Rodríguez et al., 1992). These enzymes appear to have evolved in moths to function in the production of the complex chemical signals used for sex attraction, thus contributing to the chemical diversity found in the sex pheromones of Lepidoptera. In this context, it is of interest to investigate the different functional desaturases involved in the production of complex pheromone mixtures at the molecular level. An interesting example is found in the moth *Spodoptera littoralis*. Females of this species use a mixture of several C14 acetates with different degrees of unsaturation, including tetradecyl acetate, (*Z*)-9, (*E*)-11 and (*Z*)-11-tetradecenyl acetates and (*Z*,*E*)-9,11 and (*E*,*E*)-10,12-tetradecadienyl acetates (Nesbitt et al., 1973; Tamaki and Yushima 1974; Campion et al., 1980; Dunkelblum et al., 1982; Navarro et al., 1997a). Biosynthesis of the *S. littoralis* sex pheromone blend involves desaturation of palmitic acid followed by chain shortening via β-oxidation (Martinez et al., 1990; Navarro et al., 1997b). Different desaturation reactions are involved in the biosynthetic pathways for the various components: (1) Δ^{11} desaturation of palmitic acid into (*Z*)-11-hexadecenoic acid, and of myristic acid into both (*E*)- and (*Z*)-11-tetradecenoic acids; (2) Δ^9 desaturation of (*E*)-11-tetradecenoic acid into (*Z*,*E*)-9,11-tetradecadienoic acid and (3) Δ^9 desaturation and rearrangement of (*Z*)-11-tetradecenoic acid into (*E*,*E*)-10,12-tetradecadienoic acid. Both the cryptoregiochemistries (Pinilla et al., 1999; Abad et al., 2000; Rodríguez et al., 2001) and stereoselectivities (Navarro et al., 1997b; Abad et al., 2001; Rodríguez et al., 2002) of these reactions were reported in previous articles. In this work, we report on the cloning and functional expression of the desaturases involved in some of these biotranformations.

2. Experimental procedures

2.1. Insect tissue collection and poly(A)+ RNA isolation

Insects were reared in the laboratory as reported elsewhere (Martinez et al., 1990). Fat body tissue and pheromone glands were carefully dissected from 2- to 3-day-old female moths and stored at −80 °C. Oligo-dT cellulose was used to isolate Poly(A)$^+$ RNA (mRNA) from the dissected tissues using an mRNA Isolation kit (Ambion) according to the instructions provided by the manufacturer.

2.2. Cloning of desaturase cDNA from fat body

One microliter of mRNA isolated from fat bodies was denatured and transcribed to single-stranded cDNA (3′-ss-cDNA) by reverse transcriptase with the GeneRacer™ rapid amplification cDNA ends (RACE) amplification kit (Invitrogen). This 3′-ss-cDNA was diluted (1:10; final volume, 100 µl) and used as template for polymerase chain reaction (PCR) to obtain the central region of a desaturase gene. Two degenerate primers, PR3 and PR4 (Table 1), which were designed based on highly conserved regions of desaturase genes, were used. A 50-µl standard reaction contained 25 µl of

Table 1
Primers used in this study

Abbreviation	Sequence (5′→3′)
PR1	ATCACNGCHGGBGMYCAYMG
PR2	GGRAASDYRTGRTGRWARTT
PR3	GGYATYACVGCHGGNGCWCA
PR4	TGRTARTTRTGGAABSCYTCNCC
PR5	GACCAYMGNHWSCAYCA
PR6	GGRWAVRYRTGRTGRTARTT
GR-3′-P	GCTGTCAACGATACGCTACGTAACG
GR-3′-NP	CGCTACGTAACGGCATGACAGTG
GR-5′-P	CGACTGGAGCACGAGGACACTGA
GR-5′-NP	GGACACTGACATGGACTGAAGGAGTA
CR1-5′-1	GTTTCCACCGGGTTGATGTTCTTAT
CR1-5′-2	GTAACGAAAGATGGAGCACACGAA
CR1-3′-1	GATGCATCACAAGTACTCCGAAACC
CR1-3′-2	GTATTGAACGTGACTTGGCTGGTGA
ORF1-5′	TACTCTAGACTTCTAGTTGTGCTATTACAC
ORF1-3′	ACAGAGCTCCTTCGATCTAATGGAGTTTGAC
CR2-5′-1	CTTATGAGCAGCGGAGTTGACGA
CR2-5′-2	TTTAGTGGCCATTTAGCTTTATAGGATTTG
CR2-3′-1	CTCAAGGAAAAGGGCAAAGGAC
CR2-3′-2	TCATAAGTGGGGAGACAAACCT
ORF2-5′	TTTTCTAGAATGGCTCCAAATATATCGGAGGA
ORF2-3′	TCAGAGCTCTTAATCATCCTTAGGGTTAATCCTA
CR3-5′-1	CCGATGATGGAGTGGATGGTCTTC
CR3-5′-2	GACTGTGTTTTGAAACGCAAGGGA
CR3-3′-1	GACTTTGGTCGCATAAAACTTAC
CR3-3′-2	CGTAGGATCTTTCTATTCCACATCG
ORF3-5′	ACTGGAGCTCAAAATGGCGCAATGTGTAC
ORF3-3′	ACTGTCTAGACTATTCGCCTTTTGTATTTT
CR4-5′-1	TCACCGAGAGCTGCCAAACTTACTA
CR4-5′-2	CAACAACGCGAACAATGGAATGAAGAAT
CR4-3′-1	GGAAGACCGGCGCATTGCACTAAAACC
CR4-3′-2	TCGTGAAATCGGTGGAGAATAGTC
ORF4-5′-1	AGCTTCTAGAATGGCTCCAGCGCAAAAACAC
ORF4-3′-1	AGCTGAGCTCTCATTCGGCGTGACAT
ORF4-5′-2	ACTGAGCTCACTATGGCTCCAGCGCAAAAACAC
ORF4-3′-2	AGCTTCTAGATCATTCGGCGTGACAT

HotStarTaq™ mix (QIAGEN), 0.5 μM PR3, 0.5 μM PR4 and 5 μl of diluted 3′-ss-cDNA. The PCR was performed under the following conditions: 95 °C denature, 15 min; 33 cycles of 95 °C, 30 s; 50 °C, 30 s; and 72 °C, 3 min with a final extra 10 min extension at 72 °C. The PCR product was ligated directly to a linearized pCR®4-TOPO® vector (Invitrogen) and the ligated vector was transformed into TOPO 10 cells. Colony PCR was used to screen for positive colonies containing the insert, and plasmids in selected positive colonies were amplified, purified and sequenced. The central region (Sls-CR1) (abbreviation Sls is used for *S. littoralis* to differentiate it from Sli used for *S. littura)* was obtained and compared with other central regions of desaturase genes to confirm its identity as a desaturase gene fragment.

Sequence information of Sls-CR1 was used to design gene-specific primers for RACE PCR. The first-round 3′ RACE PCR was performed with the gene-specific primer CR1-3′-1 (Table 1) and the GeneRacer™ 3′ primer GR-3′-P from the RACE cDNA amplification kit ($T_a = 57$ °C, 30 cycles). The second-round 3′ RACE PCR was performed by using the diluted first-round PCR products as templates with the nested gene-specific primer CR1-3′-2 (Table 1) and the GeneRacer™ 3′ nested primer GR-3′-NP from the kit ($T_a = 58$ °C, 30 cycles). With the same GeneRacer kit, another ss-cDNA library (5′-ss-cDNA) was constructed, which was used as template to run first-round 5′ RACE PCR by using the gene-specific primer CR1-5′-1 (Table 1) along with the GeneRacer™ 5′ primer GR-5′-P from the RACE cDNA amplification kit ($T_a = 57$ °C, 30 cycles). The first-round PCR product was then diluted and used as template to run the second-round PCR with the nested gene-specific primer CR1-5′-2 (Table 1) and the GeneRacer™ 5′ nested primer GR-5′-NP from the kit ($T_a = 58$ °C, 30 cycles). The second-round PCR products were ligated directly to pCR®4-TOPO® vector

for sequencing. By combining the 5′ fragment, central region and 3′ fragment, one full-length cDNA sequence, Sls-FL1, was obtained from the fat body cDNA libraries.

2.3. Cloning of desaturase cDNAs from pheromone glands

One microliter of mRNA isolated from pheromone glands was used to construct the 3′ and 5′ ss-cDNA libraries as described above. The 3′-ss-cDNA library and degenerate primers PR1 + PR2, PR3 + PR4 and PR5 + PR6 were used to amplify the central regions of desaturase sequences under the same conditions described above ($T_a = 45\,^{\circ}C$, 33 cycles for primers PR1 + PR2 and PR5 + PR6, and $T_a = 50\,^{\circ}C$, 33 cycles for primers PR3 + PR4). The PCR products were cloned directly to the linearized pCR®4-TOPO® vector and sequenced. Three central regions (Sls-CR2, Sls-CR3 and Sls-CR4) were obtained and they were used to design gene-specific primers for 3′ and 5′ RACE PCR.

The first-round 5′ RACE PCR was performed with the following primers: GR-5′-P and CR2-5′-1 (Table 1) for Sls-CR2 ($T_a = 57\,^{\circ}C$, 30 cycles); GR-5′-P and CR3-5′-1 (Table 1) for Sls-CR3 ($T_a = 58\,^{\circ}C$, 30 cycles) and GR-5′-P and CR4-5′-1 (Table 1) for Sls-CR4 ($T_a = 60\,^{\circ}C$, 30 cycles). The first-round PCR products were diluted and used as templates for second-round PCR with the 5′ nested primer GR-5′-NP combined with primers CR2-5′-2 (Table 1) for Sls-CR2 ($T_a = 54\,^{\circ}C$, 30 cycles), CR3-5′-2 (Table 1) for Sls-CR3 ($T_a = 60\,^{\circ}C$, 30 cycles) and CR4-5′-2 (Table 1) for Sls-CR4 ($T_a = 60\,^{\circ}C$, 30 cycles). The first-round 3′ RACE PCR was performed similarly with 3′ primer GR-3′-P combined with primers CR2-3′-1 (Table 1) for Sls-CR2 ($T_a = 60\,^{\circ}C$, 30 cycles), CR3-3′-1 (Table 1) for Sls-CR3 ($T_a = 52\,^{\circ}C$, 30 cycles) and CR4-3′-1 (Table 1) for Sls-CR4 ($T_a = 60\,^{\circ}C$, 30 cycles). The first-round PCR products were diluted and used as templates for the second-round PCR with the GR-3-NP and the primers CR2-3′-2 (Table 1) for Sls-CR2 ($T_a = 60\,^{\circ}C$, 30 cycles), CR3-3′-2 (Table 1) for Sls-CR3 ($T_a = 58\,^{\circ}C$, 30 cycles) and CR4-3′-2 (Table 1) for Sls-CR4 ($T_a = 60\,^{\circ}C$, 30 cycles). The second-round RACE PCR products were cloned into the pCR™4-TOPO™ vector for sequencing. By combining the 5′ fragment, central region and 3′ fragment, three full-length cDNA sequences, Sls-FL2, Sls-FL3 and Sls-FL4, were obtained from the pheromone gland cDNA libraries.

2.4. Functional assay of Δ^9 desaturases in the YEpOLEX expression system

Two gene-specific primers, open-reading frame ORF1–5′ and ORF1-3′ (Table 1), containing *Xba*I and *Sac*I restriction sites, respectively, were designed to amplify the ORF of Sls-FL1 from the fat body 3′-ss-cDNA library. Two other gene-specific primers, ORF2-5′ and ORF2-3′ (Table 1), having the same restriction sites as above were designed to amplify the ORF of Sls-FL2 from the pheromone gland 3′-ss-cDNA library. A single clear band for each of the ORFs was obtained. The PCR products were digested, purified by gel electrophoresis and ligated to linearized YEpOLEX plasmids, which were then transformed into TOPO 10 competent cells for positive colony screening. After plasmid mini-prep, the recombinant plasmids in positive colonies were amplified, purified and sequenced, and the consensus clones YEpOLEX-Sls-ORF1 and YEpOLEX-Sls-ORF2 were used for functional expression. The competent cells of the mutant *Ole1* yeast strain L8-14C (Stukey et al., 1990) were cultured as described (Liu et al., 1999) and transformed with YEpOLEX-Sls-ORF1 or YEpOLEX-Sls-ORF2 by the standard method (Ito et al., 1983). The transformed cells were able to grow on yeast extract/peptone/dextrose (YPD) plates without complementation with additional monounsaturated fatty acids (MUFA). Cells were inoculated into 50 ml of sterile YPD medium and grown at 30 °C overnight with shaking (300 r.p.m.) to approximately 1×10^9 cells/ml. The induced cells were transferred to a 50-ml sterile centrifuge tube and spun at $1500 \times g$ for 5 min, and the cell pellet was washed two times with 0.2% BSA solution and spun to remove as much liquid as possible. The washed yeast cells were lysed with 1 ml of yeast protein extraction reagent (YPER, Pierce) with brief vortex and agitation for 20 min at room temperature, and the cell debris was collected by centrifugation at $13{,}200 \times g$ for 2 min and extracted with 1 ml of chloroform/methanol (2:1) at room temperature for 1 h. The solvent was decanted from the debris and evaporated under nitrogen. The residue was dissolved in 1 ml of 0.5 M KOH in methanol (room temperature for 1 h), and then 1 N HCl (1 ml) was added and the resulting fatty acid methyl esters were extracted with hexane (1 ml). The solution was concentrated under nitrogen and stored at −30 °C until gas chromatography coupled to mass spectrometry (GC/MS) analysis. The double bond position in the products was determined by mass spectrometry (MS) analysis of the dimethyldisulfide (DMDS) adducts (Buser et al., 1983). Positions of the conjugated double bonds in dienes were determined by GC/MS analysis of their 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) derivatives (McElfresh and Millar, 1999).

2.5. Functional assay of the Δ^{11} desaturase in the pYES2 expression system

Two gene-specific primers, ORF3-5′ and ORF3-3′ (Table 1), containing the restriction sites *Sac*I and *Xba*I, respectively, were designed to amplify the ORF of

Sls-FL3. The PCR products were digested, purified and ligated to linearized pYES2 vectors (Invitrogen), which were then transformed into TOPO 10 competent cells for positive colony screening. After plasmid mini-prep, the recombinant plasmids were amplified, purified and sequenced. The consensus clone pYES2-Sls-ORF3 was obtained and was transformed into mutant *ΔOle/ΔElo1* yeast cells for functional expression. This mutant is defective in both the Δ^9 desaturase and the elongase 1 genes (Schneiter et al., 2000). The transformed cells were grown on selective medium (SD) plates with 20% glucose, 0.5 mM palmitoleic acid and 0.5 mM oleic acid in 1% tergitol (type NP-40, Sigma-Aldrich), at 30 °C for 48 h. A single colony from the SD plate was then inoculated into 30 ml of sterile SD medium with glucose and 0.5 mM each of palmitoleic and oleic acids in 1% tergitol and grown at 30 °C for 48 h with shaking at 300 r.p.m. The cell suspension was transferred to a 50 ml tube and spun at $500 \times g$ for 5 min. The cell pellet was washed two times with SD induction medium (10% galactose and 10% raffinose were added in place of 20% glucose), and the washed cells were inoculated into 30 ml of SD induction medium at a cell density of 1×10^7 ml, and grown at 30 °C for 65 h with shaking (300 r.p.m.). The induced cells were extracted as discussed above to obtain the fatty acid methyl esters and the DMDS adducts for GC/MS analysis.

2.6. Functional assay of the desaturase encoded by Sls-FL4

Two gene-specific primers, ORF4-5′-1 and ORF4-3′-1 (Table 1), containing *Xba*I and *Sac*I restriction sites, respectively, were designed to amplify the ORF corresponding to Sls-FL4. The PCR products were digested, purified ligated to linearized YEpOLEX vectors and transformed into TOPO 10 competent cells for positive colony screening. After plasmid mini-prep, the recombinant plasmids were amplified, purified and sequenced. The resulting consensus clone, YEpOLEX-Sls-ORF4, was transferred to *Ole1* cells for functional expression.

Additionally, two other gene-specific primers, ORF4-5′-2 and ORF4-3′-2 (Table 1), containing the *Sac*I and *Xba*I restriction sites, respectively, were used to amplify the same ORF. The PCR products were digested, purified and ligated to pYES2 vectors, which were then transformed into TOPO 10 competent cells for positive colony screening. After plasmid mini-prep, the recombinant plasmids were amplified, purified and sequenced. The resulting consensus clone pYES2-Sls-ORF4 was transferred to *Ole1* cells for functional expression.

2.7. Instrumental analysis

The methanolyzed lipidic extracts were analyzed by GC/MS, at 70 eV, on a Fisons gas chromatograph (8000 series) coupled to a Fisons MD-800 mass selective detector. The system was equipped with a non-polar Hewlett Packard HP-1 capillary column (30 m × 0.20 mm ID) using the following temperature programs: from 120 to 180 °C at 5 °C/min and then to 260 °C at 2 °C/min after an initial delay of 2 min (analysis of FAME); from 80 to 200 °C at 5 °C/min and then to 300 °C at 10 °C/min and held at 300 °C for 25 min (analysis of DMDS and MTAD adducts).

2.8. Phylogenetic analysis

The newly characterized nucleotide sequences of *S. littoralis* acyl-CoA desaturases described in this paper were compared to moth and dipteran sequences extracted from GenBank. An alignment of these genes was created from their deduced amino acid sequences using the computer program ClustalX (Thompson et al., 1997). The resulting alignment was subsequently checked for errors by visual inspection. Phylogenetic trees were reconstructed using the neighbor-joining method (Saitou and Nei, 1987) with 1500 bootstrap replicates as a measure of statistical reliability.

3. Results

3.1. Cloning and characterization of S. littoralis acyl-CoA desaturase genes

Reverse transcription–PCR was used to amplify a 557-bp fragment (Sls-CR1) by two degenerate primers from the fat body cDNA library. Comparison to the central regions of other desaturase genes showed that this fragment had high identity with other insect Δ^9 desaturases, such as those of *T. ni* (90%), *H. zea* (70%), *A. velutinana* (72%), *O. nubilalis* (69%) and *P. octo* (72%). A full-length cDNA sequence, Sls-FL1, was obtained by combining the central region Sls-CR1 along with 3′ and 5′ RACE fragments that were amplified by RACE PCR. This cDNA spans 1418 nt and contains an ORF of 1062 nt, which encodes a protein with 354 amino acids.

Three fragments of 564 bp (Sls-CR2), 569 bp (Sls-CR3) and 455 bp (Sls-CR4) were obtained from the pheromone gland cDNA library by using different degenerate primers. Sls-CR2 had high identity with the central regions of other insect Δ^9 desaturase genes (70% to *T. ni*, 85% to *A. velutinana*, 85% to *P. octo* and 92% to *H. zea*), whereas Sls-CR3 had high identity with other moth Δ^{11} desaturases (78% to *T. ni*, 71% to *A. velutinana*, 60% to *E. postvittana* and 86% to *H. zea*). Sls-CR4 had a relatively lower identity with other desaturases, although it does contain the 3-histidine boxes in the appropriate arrangement. These results

showed that these three fragments could be from desaturase genes.

Three full-length cDNAs (Sls-FL2, Sls-FL3 and Sls-FL4) were obtained from the fragments from the pheromone gland cDNA library. Sls-FL2 spans 1879 nt with an ORF of 1062 nt, which encodes a protein with 354 amino acids. Sls-FL3 spans 1470 nt with an ORF of 1017 nt, which encodes a protein with 339 amino acids. Sls-FL4 gave a cDNA that spans 1585 nt with an ORF of 1131 nt, which encodes a protein with 377 amino acids.

3.2. Heterologous functional expression of the new desaturase genes in S. cerevisiae

Z9-desaturases: The mutant *Ole1* yeast cells used for the functional assay are desaturase deficient and cannot grow if MUFA are not complemented in the medium. In this study, yeast cells transformed with the constitutive YEpOLEX-derived plasmids containing the ORF of the Z9-like desaturase either from the fat bodies or the pheromone gland grew well in YPD medium without the addition of MUFA. GC/MS analysis of fatty acid methyl esters obtained by base methanolysis of the yeast lipid extracts confirmed that YEpOLEX-Sls-ORF1 (fat body) expressed a Z9 desaturase that produced Z9–16:Acid < Z9–18:Acid in a (1:4.5) ratio and was labeled SlsZ9(18). The functional analyses also showed that YEpOLEX-Sls-ORF2 (pheromone gland) expressed a Z9 desaturase that produced Z9–16:Acid > Z9–18:Acid in a (1.5:1) ratio and was labeled SlsZ9(16). Double bond location in the reaction products was shown by MS analysis of DMDS adducts, which exhibited the characteristic Δ^9 fragment at m/z 217 (data not shown). Double bond geometry was determined by comparison of GC retention times to synthetic

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OnuZ9(18)    1    MPPQGAERDSFVLFESDAK--DPDVITHMAPQAEKRQTQIVWRNVAIFTYLHLGFLYGAY   58
HzeZ9(18)    1    MPPQGQTGGSWVLYETDAVNEDTDAPVIVPPSAEKREWKIVWRNVILMGMLHIGGVYGAY   60
SlsZ9(18)    1    MPPQGQTGGSWVLYETDAVNVDTEAPVIVPPSAEKREWKIVWRNVILMGLLHIGGVYGAY   60
EpoZ9(16)    1    MPPQGQPPAAWVLEESDATTDDKDVAVAVPPSAEKRKLSIVWRNVILFVLLHTCAVYCGY   60
AveZ9(16)    1    MAPNVTENG--VLFESDAATPDLALGTAPVEQADDSPRVYVWRNIILFAYLHLAAIYGGY   58
SlsZ9(16)    1    MAPNISEDVNGVLFESDAATPDLALARPPVQKADNKPKQLVWRNIILFAYLHLAALYCGY   60
             1    *.*:    ** *:**  *     .*:.   ****: :: ** . :**.*

OnuZ9(18)   59    LMLTTVMWKTRLFCLILYVCSGLGITAGAHRLWAHKSYKAKLPLRIILTLFNTLAFQDAV   118
HzeZ9(18)   61    LFLTTAMWRTCIFAVVLYICSGLGITAGAHRLWAHKSYKARLPLRLMLTLFNTLAFQDAV   120
SlsZ9(18)   61    LFLTTAMWRTSLFAVFLYICSGLGITAGAHRLWAHKSYKARLPLRILLTLFNTLAFQDAV   120
EpoZ9(16)   16    LFFTKAMWATKFFAFFLYLCSGLGITAGAHRLWAHKSYKARLPLRILLTLFNTIAFQDSV   120
AveZ9(16)   59    LFLFSAKWQTDIFAYFLYVASGLGITAGAHRLWAHKSYKAKWPLRVILTTFNTIAFQDSA   118
SliZ9(16)   61    LFLFSAKWQTDIFAYILYVISGLGITAGAHRLWAHKSYKAKWPLKVILIIFNTVAFQDAA   120
              *:: .. * * :*. .**: ********************: **:::* ***:****:.

OnuZ9(18)  119    VDWARDHRMHHKYSETDADPHNATRGFFFSHVGWLLVRKHPQIKAKGHTIDMSDLRADPV   178
HzeZ9(18)  121    IDWARDHRMHHKYSETDADPHNATRGFFFAHVGWLLVRKHPQIKAKGHTIDLSDLKSDPI   180
SlsZ9(18)  121    IDWARDHRMHHKYSETDADPHNATRGFFFSHVGWLLVRKHPQIKAKGHTIDLSDLKNDPI   180
EpoZ9(16)  121    LDWARDHRMHHKYSETDADPHNATRGFFFSHVGWLLVRKHPQIKAKGHTIDMSDLCSDPV   180
AveZ9(16)  119    IDWARDHRMHHKYSETDADPHNATRGFFFSHIGWLLVRKHPELKRKGKGLDLSDLYADPI   178
SliZ9(16)  121    MDWARDHRMHHKYSETDADPHNATRGFFFSHIGWLLVRKHPDLKEKGKGLDMSDLLADPV   180
              :**************************:*:*********::* **: :*:*** **:

OnuZ9(18)  179    LRFQKKYYMYLMPLICFIMPSVVPA-LWGETVWNGYFTCAVFRYVAVLNGTWLVNSWAHL   237
HzeZ9(18)  181    LRFQKKYYLFLMPLVCFILPCYIPT-LWGESLWNAYFVCSIFRYVYVLNVTWLVNSAAHL   239
SlsZ9(18)  181    LRFQKKHYLILMPLVCFILPCYIPT-LWGESLWNAYFVCSIFRYVYVLNVTWLVNSAAHL   239
EpoZ9(16)  181    LRFQKKYYLTLMPLFCFILPTYIPT-LWGESLWNAYFVAAIFRYCYVLNVTWLVNSAAHK   239
AveZ9(16)  179    LRFQKKYYLLLMPLACFILPTVIPVYLWNETWTNAFFVAALFRYTFILNVTWLVNSAAHK   238
SlsZ9(16)  181    LRFQKKYYLLLMPLACFVMPTMIPVYLWGETWTNAFFVAAMFRYAFNLNVTWLVNSAAHK   240
              ******:*: **** **:** :*. **.*: *.:*..::***  ** ****** **

OnuZ9(18)  237    WGDKPYDRHINPVETKVVSVAAIGEGFHNYHHTFPWDYKAAELGNYTFNITKFFIDTMAT   297
HzeZ9(18)  240    WGAKPYDKNINPVETRPVSLVVLGEGFHNYHHTFPWDYKTAELGDYSLNLTKLFIDTMAA   299
SlsZ9(18)  240    WGAKPYDKNINPVETKPVSLVVLGEGFHNYHHTFPWDYKTTELGDYPLNLTKLFIDFMAA   299
EpoZ9(16)  240    WGDRPYDKNINPVETKPVSLVVFGEGFHNYHHTFPWDYKTAELGGYSLNISKLFIDTMAK   299
AveZ9(16)  238    WGDKPYDKSIKPSENMSVSLFAFGEGFHNYHHTFPWDYKTAELGNHRLNFTTKFINFFAK   298
SlsZ9(16)  241    WGDKPYDKSIKPSENMSVAMFALGEGFHNYHHTFPWDYKTAELGNNKLNFTTAFINFFAK   300
              ** :***: *:* *. *:: .:*****************::***. .:*::. **: :*

OnuZ9(18)  298    IGWAYDLKTVSTDVIQKRITRTGDGSHPVWGWGDKNIPEEDKKVTTVINPEKEE    351
HzeZ9(18)  300    IGWAYDLKTVSTDVIQKRVKRTGDGSHPVWGWDDHEVHQADKKLAAIINPEKTE    353
SlsZ9(18)  300    IGWAYDLKTVSSDVIQKRVKRTGDGSHAVWGWDDHEVHQEDKELAAIINPDKTE    353
EpoZ9(16)  300    IGWAYDLKSVSPDIVEKRVKRTGDGSHEVWGWDDKDVPAEQKAAATIINPEKTE    353
AveZ9(16)  299    IGWAYDMKTVSNEIVQQRVKRTGDGSHHLWGWGDKDHAQEEINAAIRIHPKDD-    351
SlsZ9(16)  301    IGWAYDMKTVSEDIVKNRVKRTGDGSHHLWGWGDENQPKEEIEAATRINPKDD-    353
             *******:*:** :::::*:.******* :***.*.:  :  : *:*..
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(A)

Fig. 1. Comparison of the amino acid sequences of different desaturases. The comparisons correspond to: (A) SlsZ9(18) and SlsZ9(16) to those of known Δ^9 desaturases, (B) SlsZ/E11 to those of reported Δ^{11} desaturases and (C) Sls-NF to the partial sequences of acyl-CoA desaturase genes of *S. litura* (Accession AF482943), *S. exigua* (Accession AF482937) and *H. assulta* (Accession AF482907). Gaps in alignments are indicated by dashes. Identical amino acids are indicated by *. Two or one point below residues indicate decreasing amino acid similarity. The three histidine domains are indicated in bold. The transmembrane loops (http://www.mbb.ki.se/tmap) are shown with a waved line. The endoplasmic reticulum membrane retention signals are underlined. Sequences were obtained from GenBank and Accession numbers are: OnuZ9, AF430246; HzeZ9, AF272345; EpoZ9, AY061988; AveZ9, AF243046; TniZ11, AF035375; CroZ/E11, AF518014; OnuZ/E11, AF441221; EpoE11, AY049741; AveZ/E11, AF416738; HzeZ11, AF272342. Moth species are: *C. rosaceana*, Cro; *O. nubilalis*, Onu; *E. postvittana*, Epo; *A. velutinana*, Ave; *H. assulta*, Hass; *H. zea*, Hze; *T. ni*, Tni; *S. littoralis*, Sls; *S. exigua*, Sex; *S. litura*, Sli.

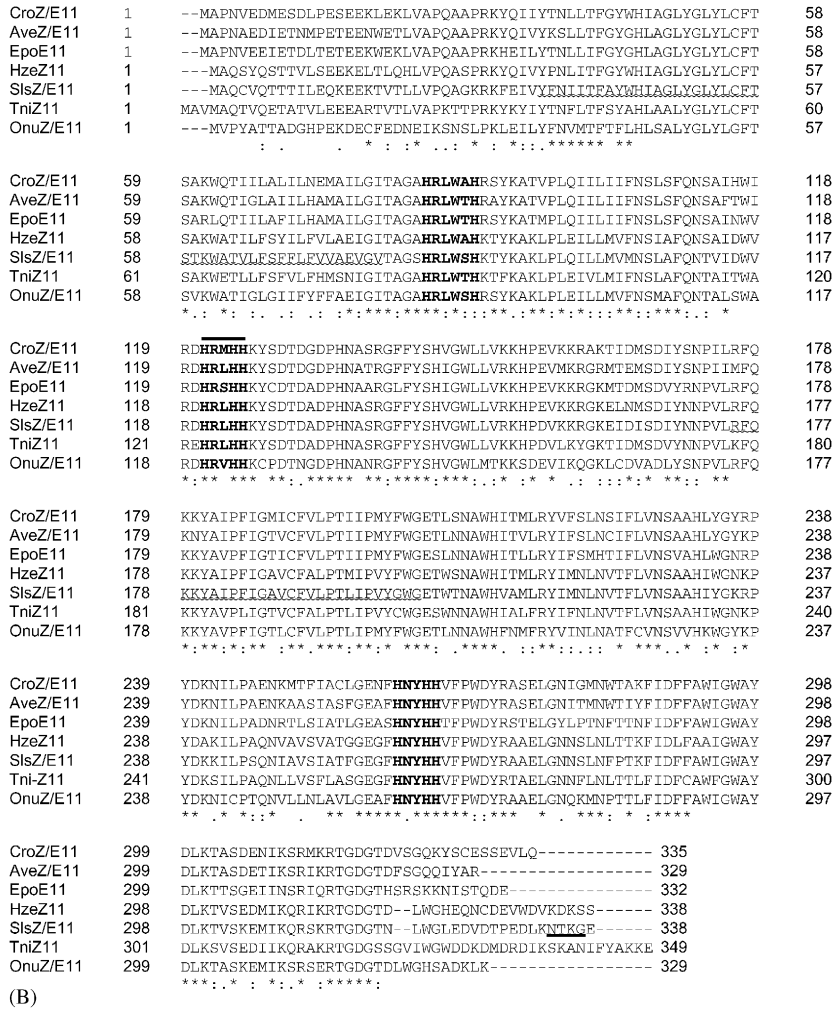
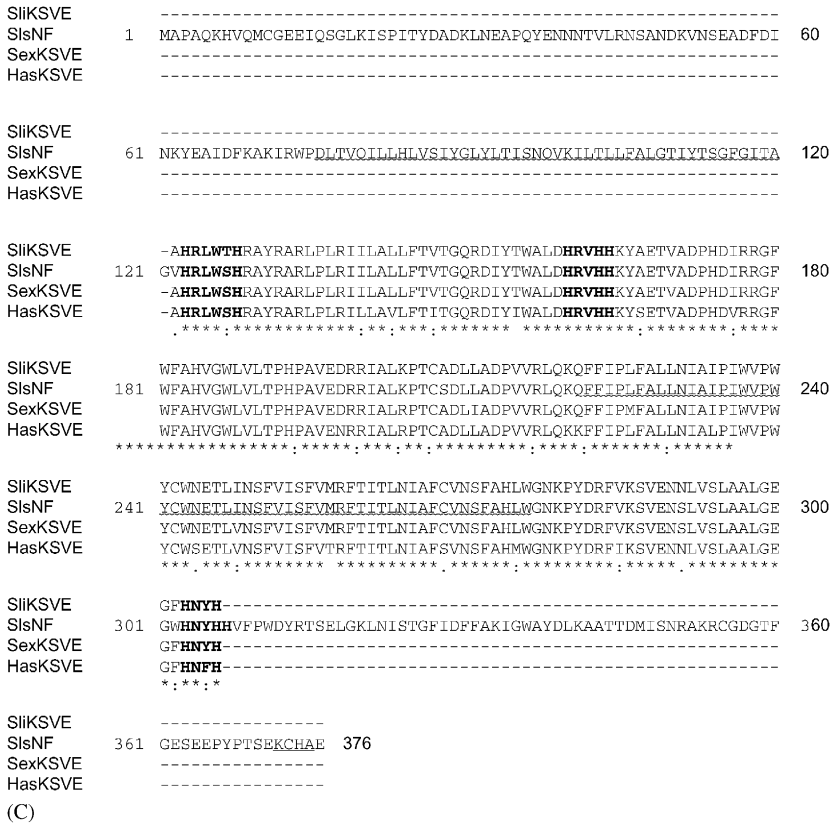


Fig. 1. (*Continued*)

standards. Comparison of the deduced amino acids of these two Z9 desaturases to those of other Z9 desaturases is shown in Fig. 1A.

Both transformed cells produced Z9–14:Acid from exogenously added 14:Acid. In contrast, when a mixture of 14:Acid and E11–14:Acid (1:1) was added to the culture medium, no methyl (*Z*)-9-tetradecenoate was detected in the methanolyzed lipidic extracts from either transformed yeast. However, a 14-carbon acid conjugated diene was produced (Fig. 2) in both transformants when 14:Acid and E11–14:Acid were added. GC/MS analysis of MTAD-derivatized samples showed that the product was Z9,E11–14:Acid (Fig. 3). The adduct exhibited a molecular ion at m/z 351 and major ions at m/z 196 and 322, which are indicative of a 9,11-conjugated system of a 14-carbon methyl ester. The GC retention time was the same as that of the *Z*,*E*-isomer adduct when compared with the adduct of the four synthetic diene isomers. SlsZ9(18) produced more of the Z9,E11–14:Acid product than did SlsZ9(16), and the former produced less of the chain elongation product E13–16:Acid than the latter. Upon addition of either E11–14:Acid or Z11–14:Acid to the yeast medium, the ratios Z9–16/Z9–18 changed from 1:4.5 to 1:9 with SlsZ9(18) and from 1.5:1 to 1:1 with SlsZ9(16) as compared to the ratios found in cells grown in the absence of the added C14 MUFA.

SlsZ/E11: The pYES2 expression system was used for the functional analysis of pYES2-Sls-ORF3. Preliminary experiments using the mutant *Ole1* yeast cells gave very low amounts of Δ^{11} unsaturated products and a different yeast mutant defective in both the Δ^{9} desaturase and the elongase 1 genes was used. Transformation of this strain with the inducible pYES2-derived plasmid containing the Sls-FL3 ORF produced a mixture of E11–14:Acid, Z11–14:Acid, Z11–16:Acid and *Z*-11–18:Acid in a 5:4:60:31 ratio (Fig. 4). The function of the gene product as a Δ^{11}desaturase was proven by GC/MS analysis of the DMDS derivatives of the fatty acid methyl esters obtained by methanolysis of the yeast lipid extracts. The mass spectra of the DMDS adducts exhibited the characteristic Δ^{11} fragment at m/z



(C)

Fig. 1. (*Continued*)

245 (Fig. 5). The double bond configuration was determined by comparison of GC retention times to synthetic standards. This pheromone gland desaturase was named SlsZ/E11. Comparison of the deduced amino acids of this desaturase with several other Δ^{11} desaturases is given in Fig. 1B.

Attempts of functional expression *of the SlsNF gene*: In the first attempt, the Sls-ORF4 inserted into YEpOLEX failed to complement the MUFA auxotrophy of the *Ole1* mutant yeast. Therefore, transformed cells were grown in YPD medium containing both palmitoleic and oleic acids. No distinctive products were detected after incubation of yeast in the presence of C14:Acid, Z9–14:Acid, E11–14:Acid, Z11–14:Acid or Z11–16:Acid.

The same results were obtained when the pYES2 expression vector was used with Sls-ORF4. The addition of various precursors, such as C14:Acid, Z9–14:Acid, E11–14:Acid, Z11–14:Acid or Z11–16:Acid, did not give evidence for the occurrence of any product not also present in controls. Since no function was discovered for this desaturase, it was named SlsNF. Nevertheless, it is possible that the appropriate substrate was not found in this study and, therefore, that SlsNF is a nonfunctional enzyme cannot be claimed. Comparison of the deduced amino acids of this gene with other published nonfunctional desaturases is given in Fig. 1C.

4. Discussion

Four desaturase-like genes were cloned from pheromone gland and fat body tissue of *S. littoralis*. The deduced amino acid sequences of these genes exhibit three histidine-rich sequences in the conserved arrangement characteristic of membrane-bound desaturases (Wada et al., 1993), as well as two significant hydrophobic stretches that span the membrane twice (Fig. 1). The position and length of the transmembrane stretches relative to the conserved histidine boxes are similar to those of other membrane-bound desaturases. The proteins also contain endoplasmic reticulum membrane retention signals in the C-terminus (Fig. 1). Thus, these proteins conform to the model proposed by Stukey et al. (1990), in which the peptide chain spans the membrane four times and exposes the three histidine clusters on the cytoplasmic side of the endoplasmic reticulum. In addition, the newly characterized *S. littoralis* genes share high levels of deduced amino acid sequence identity with certain partially sequenced *S. littura* genes identified by Knipple et al. (2002) (Fig. 6).

As discussed above, two Δ^{11} desaturation reactions occur in *S. littoralis* pheromone glands: transformation of palmitic acid into Z11–16:Acid and that of myristic acid into both Z11- and E11–14:Acid. In a previous article (Pinilla et al., 1999), we reported that

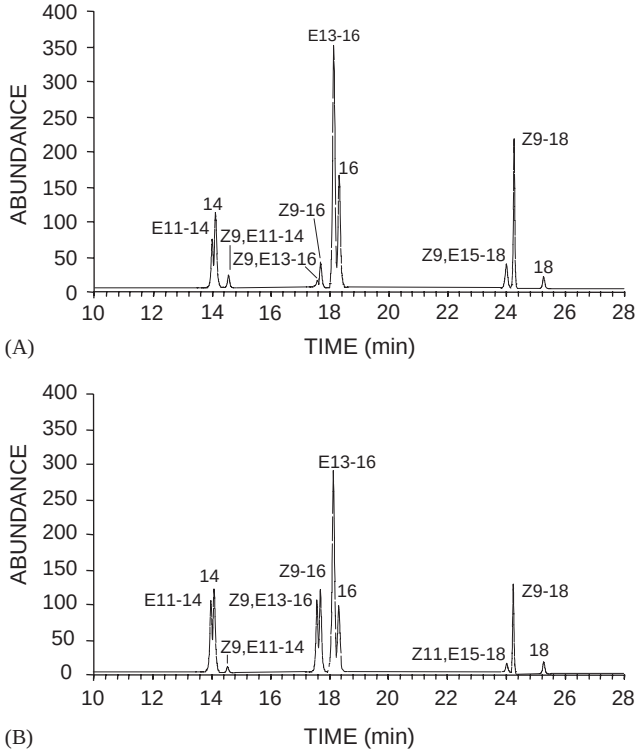


Fig. 2. GC/MS total ion chromatograms of lipid extracts from yeasts transformed with Δ^9 desaturases. The traces correspond to methanolyzed lipidic extracts from yeast cells transformed with the YEpOLEX-Sls-ORF1 (A) and YEpOLEX-Sls-ORF2 (B) constructs incubated in the presence of E11–14:Acid. The experiments were performed as described in the experimental section. 14, methyl myristate; E11–14, methyl (*E*)-11-tetradecenoate; Z9,E11–14, methyl (*Z,E*)-9,11-tetradecadienoate; Z9,E13–16, methyl (*Z,E*)-9,13-hexadecadienoate; Z9–16, methyl (*Z*)-9-hexadecenoate; E13–16, methyl (*E*)-13-hexadecenoate; 16, methyl palmitate; Z11,E15–18, methyl (*Z,E*)-9,15-octadecenoate; Z9–18, (*Z*)-9-octadecenoate; 18, methyl octadecanoate.

pentadecanoic and heptadecanoic acids were transformed into (*Z*)-11-pentadecenoic and (*Z*)-11-heptadecenoic acids, respectively, whereas both tetradecanoic and tridecanoic acids were converted to a mixture of Z11 and E11 isomers. In light of these overall results, we proposed that these Δ^{11} desaturation reactions could be catalyzed by a single enzyme. This assumption has been confirmed in this work by the yeast functional assay with SlsZ/E11. Similar results had been already found in *A. velutinana* (Liu et al., 2002b) and *C. rosaceana* (Hao et al., 2002b), in which a single desaturase produces both Z11- and E11–14:Acid, and in *O. nubilalis* where a single

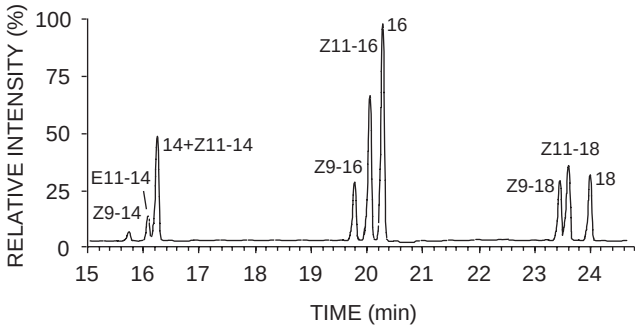


Fig. 4. GC–MS total ion chromatograms of lipid extracts from yeasts transformed with a Δ^{11} desaturases. Traces correspond to fatty acid methyl esters obtained by methanolysis of lipidic extracts from yeast cells transformed with the pYES2-Sls-OR3 construct. The experiments were performed as described in the experimental section. 14, methyl myristate; Z9–14, methyl (*Z*)-9-tetradecenoate; E11–14, methyl (*E*)-11-tetradecenoate; Z11–14, methyl (*Z*)-11-tetradecenoate; Z9–16, methyl (*Z*)-9-hexadecenoate; Z11–16, methyl (*Z*)-11-hexadecenoate; 16, methyl palmitate; Z9–18, (*Z*)-9-octadecenoate; Z11–18, methyl (*Z*)-11-octadecenoate; 18, methyl octadecenoate.

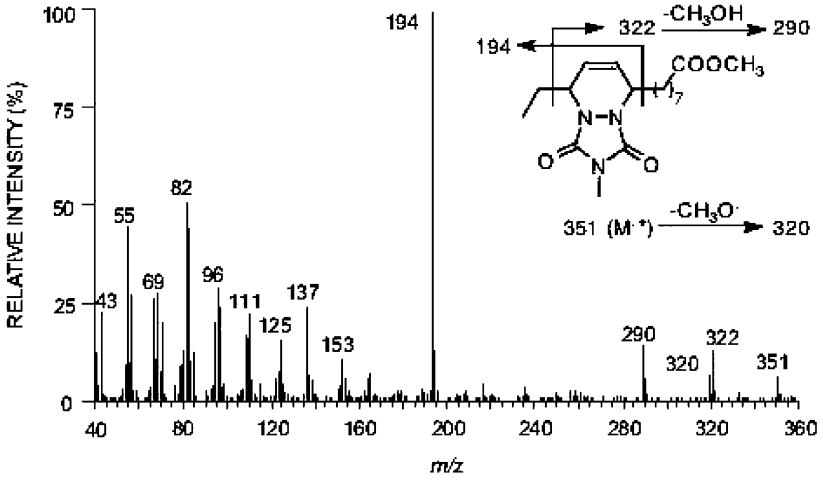


Fig. 3. Mass spectrum of the MTAD adduct of methyl (*Z,E*)-9,11-tetradecadienoate.

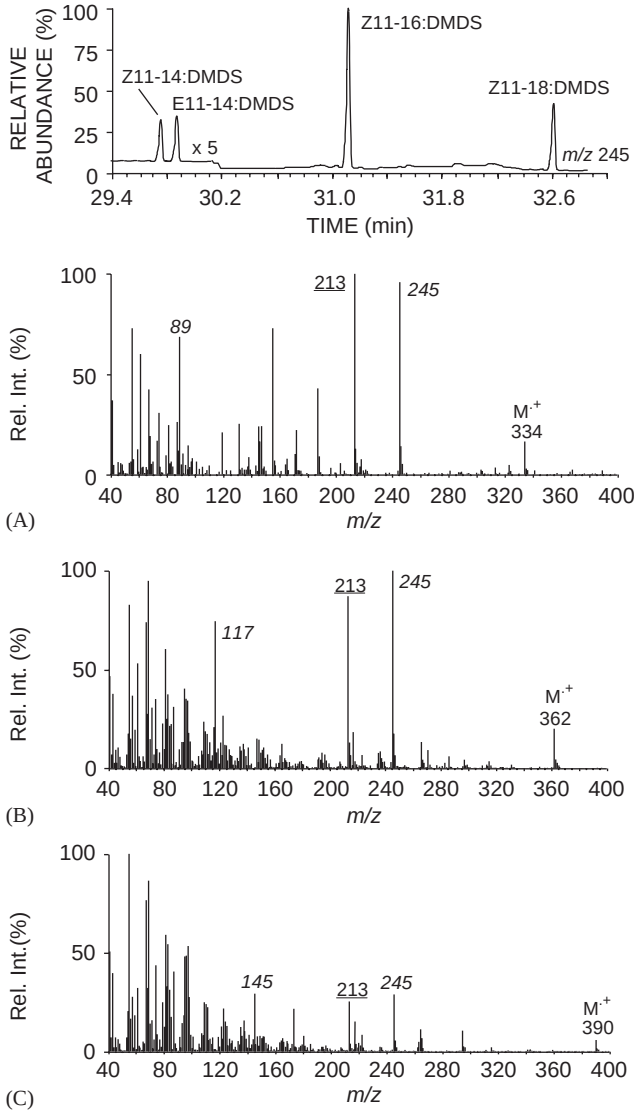


Fig. 5. Analysis of DMDS adducts. GC–MS chromatogram obtained by selection of the ion at *m/z* 245 in the analysis of the DMDS adducts of methyl esters recovered from yeast cells transformed with the pYES2-Sls-ORF3 construct. The different DMDS adducts are abbreviated as: E11–14:DMDS, dimethyldisulfide adduct of methyl (*E*)-11-tetradecenoate; Z11–14:DMDS, dimethyldisulfide adduct of methyl (*Z*)-11-tetradecenoate; Z11–16:DMDS, dimethyldisulfide adduct of methyl (*Z*)-11-hexadecenoate; and Z11–18:DMDS, dimethyldisulfide adduct of methyl (*Z*)-11-octadecenoate. The MS spectra of compounds labeled as: E11–14:DMDS (A), Z11–16:DMDS (B) and Z11–18:DMDS (C) are shown below the chromatogram. The MS spectrum of Z11–14:DMDS is identical to that of E11–14:DMDS and is not shown. The diagnostic ions are indicated in italics; the ion indicated with the underlined label arises from loss of methanol from the methyl ester containing diagnostic fragments.

enzyme produces Z11–16:Acid as well as a mixture of Z/E11–14:Acid (Roelofs et al., 2002).

Two Δ^9 desaturases have also been cloned and characterized from *S. littoralis* fat bodies and pheromone gland. From the examples so far reported, it appears that the fat body Δ^9 desaturase of all insects studied, except noctuids, is similar to that of other organisms in producing more palmitoleic than oleic acid. However, in the noctuids, a Δ^9 desaturase is found in the fat body that uses stearic preferentially over palmitic acid. Genes that code for this type of desaturase form the Δ^9 ($18>16$) group, which represents a more recently derived group of Δ^9 desaturases (Roelofs and Rooney, 2003). The Δ^9 desaturases cloned in this work conform to this general rule since the fat body enzyme of the noctuid, *S. littoralis*, produces more oleic than palmitoleic acid (Z9–16:Acid/Z9–18:Acid $\approx$ 1:4.5), whereas the pheromone gland enzyme synthesizes more palmitoleic than oleic acid (Z9–16:Acid/Z9–18:Acid $\approx$ 1.5:1).

The two enzymes, SlsZ9(16) and SlsZ9(18), produce Z,E-9,11–14:Acid when yeast is grown in the presence of E11–14:Acid. Similar results were reported (Liu et al., 2002a) for the E11-desaturase of *E. postvittana*, in which a single desaturase produces (*E*)-11-hexadecenoic acid, E11–14:Acid and (*E,E*)-9,11-tetradecadienoic acid. In this case the diene is produced by E11-desaturation of (*E*)-9-tetradecenoic acid, which is present due to chain shortening of the (*E*)-11-hexadecenoic acid. These examples show that fatty acyl-CoA esters containing *E* double bonds, such as E11–14:Acid and (*E*)-9-tetradecenoic acid, are structurally similar to saturated precursors and can be used by desaturases. SlsZ9(18) was more effective in producing the Z,E-9,11–14:Acid in yeast, which might indicate involvement of this Z9-desaturase in the insect gland to produce this pheromone precursor.

As mentioned above, both SlsZ9(16) and SlsZ9(18) produced Z9–14:Acid from 14:Acid, but no Δ^9 desaturation of 14:Acid occurred when either E11- or Z11–14:Acid was supplemented in the medium. These results would explain why in the biosynthesis of *S. littoralis* sex pheromone, Z9–14:Acid is generated by β-oxidation of Z11–16:Acid and not by SlsZ9(16)-catalyzed Δ^9 desaturation of 14:Acid (Martinez et al., 1990), which would be inhibited or down-regulated by the 11-tetradecenoates present in the gland. In this regard, it has been reported that unsaturated fatty acids inhibit the expression of the Δ^9 stearoyl-CoA desaturase gene (Ntambi and Bene, 2001; Martin et al., 2002). Also, both Δ^9 stearoyl-CoA (Park et al., 2000; Choi et al., 2001) and Δ^5 and Δ^6 linolenoyl-CoA desaturase activities (Eder et al., 2002) are inhibited by unsaturated fatty acids. In the case of SlsZ9(16), quantification of the desaturase transcripts and their expression levels is necessary to clarify if the 11-tetradecenoates effect occurs at the protein or the gene level.

In a previous article (Rodríguez et al., 2002) we proposed that formation of Z9,E11–14:Acid from E11–14:Acid and of (*E,E*)-10,12-tetradecadienoic acid from Z11–14:Acid might be catalyzed by the same

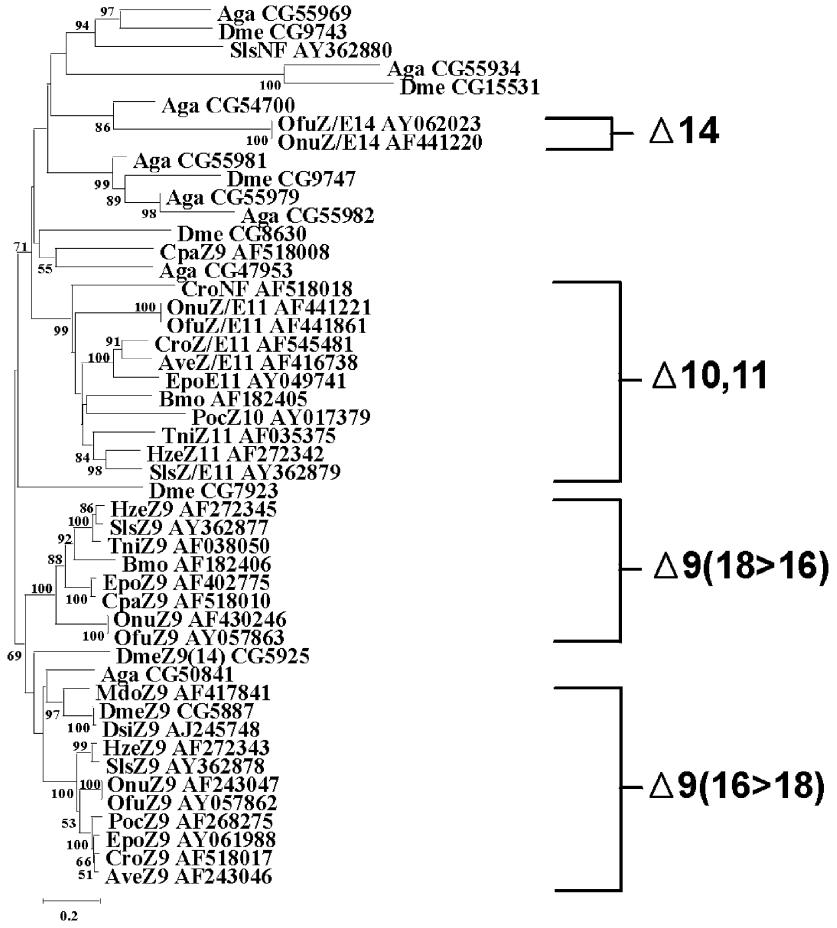


Fig. 6. Phylogeny of the desaturase genes of various moth and dipteran species. The computer program MEGA2 (Kumar et al., 2001) was used to reconstruct the tree from deduced amino acid sequences using the neighbor-joining method (Saitou and Nei, 1987). Numbers along branches indicate bootstrap support from 1500 replicates. The accession numbers for sequences are given after the species abbreviations, which follow previously published studies (Roelofs et al., 2002; Roelofs and Rooney, 2003). The newly characterized sequences from *S. littoralis* are abbreviated as "Sls", and the sequences from *S. littura,* previously characterized by Knipple et al. (2002), are abbreviated "Sli". Note that the latter have not been biochemically characterized. In the case of *D. melanogaster,* the numbers following the species abreviation are the gene designations found in the complete genome sequence database, which is accessible via GenBank. The tree is rooted on the Δ(16>18) group, based on the results of previous studies (Roelofs et al., 2002; Roelofs and Rooney, 2003).

desaturase. However, the absence of the expected E10,E12-diene in both Δ^9 desaturase yeast transformants incubated with Z11–14 does not support this hypothesis. However, production of the E10,E12 compound involves a rearrangement after desaturation that may not be possible in the yeast expression system.

No function could be found from Sls-NF gene in either the YEpOLEX or the pYES2 yeast expression system with various fatty acids added. Although the clone has all the characteristics of a desaturase gene, it does not appear to produce an active enzyme. It does have high homology with the predicted partial sequences of acyl-CoA desaturase genes of *S. litura* (Accession AF482943), *S. exigua* (Accession AF482937) and *H. assulta* (Accession AF482907) (Jeong et al., 2003; Knipple et al., 2002). Jeong et al. (2003) proposed that these three proteins are nonfunctional genes that have been maintained over the evolutionary histories of the species in which they were found. In fact, the nonfunctional *S. littoralis* desaturase gene (designated "NF") clustered with the *Drosophila melanogaster* gene CG9743, one of the dipteran desaturase homologs of unknown function (Roelofs et al., 2002; Roelofs and Rooney, 2003), suggesting that the two genes are orthologous and represent the same gene. If this is true, it would, in turn, indicate that this gene has been maintained in moths and dipterans at least since they last shared a common ancestor over 300 million years ago.

It has been suggested (Jeong et al., 2003) that the very low amounts and rarity in pheromone glands of the transcripts encoding nonfunctional desaturases indicate that they are unlikely to be involved in sex pheromone biosynthesis but that they might have functional roles in

other tissues or biochemical pathways. It has also been suggested (Roelofs and Rooney, 2003) that nonfunctional desaturase genes might represent either pseudogenes or genes that are epigenetically repressed or possibly down-regulated for some reason yet to be determined. Clearly, these are important questions to resolve. Interestingly, other studies show that acyl-coA desaturases share structural similarities to omega-hydroxylases (Shanklin et al., 1997; Broadwater et al., 2002; Shanklin and Whittle, 2003). Thus, the possibility that nonfunctional desaturase genes represent functional hydroxylases is an intriguing hypothesis worthy of future investigation. For the time being, however, we note that the term "nonfunctional" reflects that the proteins lack desaturase activity, leaving open the possibility that the proteins might be engaged in a function other than desaturation.

Roelofs and Rooney (2003) suggested that computational and phylogenetic analyses could be used to predict the functions of genes that have been assigned as putative desaturases. In our study, we showed that the biochemical activities of the *S. littoralis* desaturases follow what would be predicted on the basis of the functional groups in which they cluster (Fig. 6). Thus, our study confirms that these in silico approaches can indeed accurately predict the function(s) of newly sequenced desaturase genes. We suggest that if a desaturase clusters within one of the functional groups shown in Fig. 6 and in previous studies (Roelofs et al., 2002; Jeong et al., 2003) with 90% or greater bootstrap support, it should be considered a member of that group and highly likely to possess desaturase activity. However, we caution that biochemical characterization is still important, particularly in the case of genes falling within the Δ10,11 group, as they can possess various types of enzymatic activities (e.g., Z10, Z11, Z/E11, E11). In addition, certain desaturase genes may not fall within any of these groups but may possess a previously unknown desaturase activity. The latter could only be characterized through the use of biochemical functional assays. Regardless, the computational/phylogenetic method that we have suggested here and elsewhere (Roelofs and Rooney, 2003) should prove useful to researchers characterizing new desaturase genes or proteins.

Acknowledgments

We thank Prof. Charles E. Martin for kindly providing the yeast L8-14C strain and also for his advice and Prof. Sepp Kohlwein for generously furnishing the Δ*Ole*/Δ*Elo1* double mutant strain. The excellent technical assistance of Marion O'Connor is gratefully acknowledged. We also thank the Generalitat de Catalunya for the pre-doctoral fellowship to S. Rodríguez and for financially supporting her 4-month sojourn in Geneva. This work was supported by the MCYT, Plan Nacional de I+D (Grant AGF 2001-0585), Generalitat de Catalunya (Grant 2001 SGR-00342) and FEDER funds from EU (to G.F. and F.C.).

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